Duodenal mucosal reductase in wild-type and Hfe knockout mice on iron adequate, iron deficient, and iron rich feeding


Background: Genetic haemochromatosis is a common hereditary iron loading disorder in humans. The disease is associated with loss of function mutations in the HFE gene. This is thought to change iron stores via increased iron absorption.

Aims: In this study we investigated how adaptation of mucosal reductase activity is engaged in this process and how the changes compare with adaptation seen when an iron deficient diet is fed.

Methods: Duodenal mucosal surface reductase was measured with nitroblue tetrazolium in age matched groups of male Hfe knockout mice (Hfe) and wild-type mice fed a purified diet containing normal (iron adequate), high (iron rich), or low (iron deficient) iron concentrations.

Results: Reductase activity increased when mice were fed an iron deficient diet and decreased when they were fed an iron rich diet. Total villus activity, as measured by the average area under the activity curve along the crypt-villus axis, was increased 2.8–2.9-fold by iron deficiency in both genotypes. Approximately half of this difference was attributable to the significantly increased length of the villus in mice on an iron deficient diet (p<0.05). Hfe knockout did not affect villus length but increased mucosal reductase activity near the villus tips. Similar increases (1.3–1.6-fold) were seen on all diets but the increase was significant for iron deficient and iron loaded diets only (p<0.05).

Conclusion: Hfe gene product and dietary iron downregulate villus reductase activity in mice.
warm (37°C) 150 mM NaCl, blotted, and gently wiped to remove mucus and food particles. The duodenum adjacent to the pylorus was cut transversally to give a 1–2 mm wide slice. Duodenal fragments were incubated in vitro with 1 mM NBT in 125 mM NaCl, 16 mM HEPES-Na, pH 7.4, 3.5 mM KCl, and 10 mM glucose for five minutes at 37°C, rinsed in 150 mM NaCl, imaged (Polaroid Microcam, dissecting microscope), and scanned with a Biorad densitometer (Molecular Analyst software). Five well orientated representative villi were scanned in each piece of tissue.

Statistics
Data were averaged to give a villus length and optical density profile for each mouse. Data for groups of mice were then averaged in a similar manner. Results are given as mean (SEM). Multiple group comparisons were performed by ANOVA after testing for normal distribution. Two corresponding groups were compared by unpaired Student’s t test.

RESULTS
Mice are well able to regulate iron absorption to maintain iron homeostasis in the face of wide variations in dietary iron concentration. The data suggest that this is also true in Hfe KO mice. In contrast, there were significant differences in tissue iron load between WT and Hfe KO mice. Deletion of the Hfe gene protected mice against iron deficiency when they were fed an iron deficient diet (table 1). On the other hand, different dietary iron levels led to significant differences in tissue iron concentrations. The dietary effects were similar in WT and Hfe KO animals however (table 1). NBT staining due to deposition of the reduced product NBT formazan on duodenal villi was measured. Representative samples from some of the experimental groups are shown in fig 1. Figure 2 shows NBT staining data averaged for 7–13 WT mice on the three different diets. Reductase activity increased on an iron deficient diet and decreased on an iron rich diet. A significant increase in villus length was noticeable in iron deficient diet fed mice (table 2). There was a tendency for villus length to decrease with iron rich diet feeding but this did not reach significance. Iron deficient diet feeding produced large increases in reductase activity. Total villus activity, as measured by the average area under the activity curve, was increased 2.8–2.9-fold by iron deficiency in both genotypes. Approximately half of this difference was attributable to the increased length of the villi (p<0.05) (see fig 2, table 2), seen when an iron deficient diet was fed.

Figure 3A–C shows increased activity near the villus tips when Hfe is knocked out. Similar (1.3–1.6-fold) increases were seen on all diets but the increase was significant for iron deficient and iron loaded diets only (p<0.05). Hfe KO did not significantly affect villus length (table 2). The increase in

| Table 1 Tissue iron and haemoglobin concentrations in mice |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | Liver (nmol/mg) | Pancreas (nmol/mg) | Spleen (nmol/mg) | Kidney (nmol/mg) | Hb (g/dl)       |
| WT iron deficient | 0.50 (0.04)     | 0.58 (0.08)     | 1.08 (0.10)     | 0.65 (0.10)     | 15.4 (0.4)      |
| WT iron adequate | 1.08 (0.09)     | 0.62 (0.11)     | 5.32 (0.58)     | 0.79 (0.09)     | 15.7 (0.3)      |
| WT iron rich    | 16.5 (0.6)      | 0.93 (0.03)     | 10.44 (0.97)    | 1.68 (0.11)     | 16.8 (0.4)      |
| Hfe iron deficient | 2.25 (0.22)    | 1.11 (0.14)     | 2.46 (0.15)     | 0.92 (0.13)     | 16.9 (0.4)      |
| Hfe iron adequate | 6.07 (0.53)    | 1.04 (0.11)     | 6.72 (0.50)     | 1.39 (0.14)     | 17.2 (0.3)      |
| Hfe KO iron rich | 24.3 (1.10)    | 2.13 (0.30)     | 12.35 (1.23)    | 2.30 (0.27)     | 16.8 (0.4)      |

ANOVA
Effect of diet <0.001 <0.001 <0.001 <0.001 0.254
Hfe KO <0.001 <0.001 <0.001 <0.001 0.254

Mouse groups are: WT, wild-type; Hfe, Hfe knockout.
Data are mean (SEM) for n=10–55 mice.
The bottom rows show the p values for the effects of diet or Hfe knockout (KO) by analysis of variance (ANOVA).
reductase seen along the lower 0.3 mm of villi was similar when dietary iron was increased or decreased or when the Hfe gene was knocked out, in that a broadly similar proportional increase was observed at all points along the villus. Iron deficient diet feeding had an additional effect of increasing villus length with an associated further increase in total reductase activity.

**DISCUSSION**

Hfe KO mice have been validated previously as a model of genetic haemochromatosis. Increased duodenal iron uptake and absorption of dietary iron is strongly implicated as the main cause for increased iron storage in Hfe KO mice. Previous studies have provided contradictory findings as to whether mucosal surface ferric reductase activity is increased when HFE gene function is compromised. Dupic and colleagues showed that expression of the putative ferric reductase gene Dcytb is increased in Hfe KO mice but their study did not investigate how these changes are distributed along the villus. The present work is the first attempt to quantify changes in villus reductase along the duodenal crypt-villus axis. We have shown that the maximum activity of reductase occurs near the tip—that is, further from the crypt than the maximum level of iron uptake (Debnam E et al., unpublished observations in mice). The difference in distribution of the two activities reflects a difference in distribution of the proteins DMT1 and Dcytb. This distribution may make sense considering that trivalent dietary iron will pass through the unstirred water layer at the tip where it is reduced to Fe²⁺ and can be taken up by the DMT-1 carrier function further down the villus.

The increase in reductase activity begins low on the villus in iron deficient diet fed mice, with a significant increase detected within 50 µm of the villus base. The decrease in activity seen with an iron rich diet also extends towards the villus base. This is consistent with the prevailing hypothesis regarding control of iron absorption by iron stores—that is, that changes in iron requirements are detected in the crypt, resulting in maturing villus enterocytes that display altered absorption genes causes increased iron absorption after a lag time necessary to repopulate the villus with these adapted enterocytes.

Hfe KO did not alter the pattern of change seen in iron deficient mice. Similarly, changes in dietary iron concentration did not alter the effect of Hfe KO, although the effect of Hfe KO on mucosal reductase only achieved statistical significance in mice fed iron deficient or iron rich diets. The study of Griffiths and colleagues failed to find a significant increase in reductase activity in Hfe KO mice, consistent with our findings in mice fed an iron adequate diet. The fact that only small increases in reductase activity were seen (less than twofold) is likely due to the use of the B6 background strain in our study. This background strain has been shown to give a smaller effect of Hfe KO than other strains. The use of this strain has the advantage however that massive changes in iron stores are avoided, thus they can be more useful for mechanistic studies of Hfe function.

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**Table 2** Villus length in mouse duodenum

<table>
<thead>
<tr>
<th>Group</th>
<th>Villus length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT iron deficient</td>
<td>393 (12) (13)*</td>
</tr>
<tr>
<td>WT iron adequate</td>
<td>317 (24) (8)</td>
</tr>
<tr>
<td>WT iron rich</td>
<td>286 (12) (8)</td>
</tr>
<tr>
<td>Hfe iron deficient</td>
<td>404 (28) (10)*</td>
</tr>
<tr>
<td>Hfe iron adequate</td>
<td>331 (15) (8)</td>
</tr>
<tr>
<td>Hfe iron rich</td>
<td>313 (17) (7)</td>
</tr>
</tbody>
</table>

Mouse groups are: WT, wild-type; Hfe, Hfe knockout.

Data are mean (SEM).

*p<0.05 versus appropriate iron adequate diet

Significantly increased (p<0.05) in the region 0.29–0.37 mm of the iron deficient diet Hfe KO villi compared with wild-type mice and between 0.25–0.30 mm in iron rich diet Hfe KO villi versus wild-type mice.”

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**Figure 2** Villus reductase activity in mice. Nitroblue tetrazolium (NBT) stained villi were scanned for NBT formazan. Optical density profiles [in arbitrary units (au)] were obtained by averaging five villi per mouse, then 7–13 mice per experimental group. Profiles are for wild-type mice fed on different diets. Profiles show mean (SEM) optical density at the given points along the villus. p<0.01, iron deficient diet versus iron adequate diet [area under the curve].

**Figure 3** Effect of Hfe knockout (KO) on villus reductase activity. (A) Iron adequate diet, (B) iron deficient diet, and (C) iron rich diet. Optical density [in arbitrary units (au)] was significantly raised (p<0.05) in the region 0.29–0.37 mm of the iron deficient diet Hfe KO villi compared with wild-type mice and between 0.25–0.30 mm in iron rich diet Hfe KO villi versus wild-type mice. *Significantly increased (p<0.05).
Iron deficient diet feeding was found to increase villus length, as has been shown in rats. Feeding an iron rich diet caused a small non-significant reduction in villus length. Oates and colleagues reported that iron rich diets such as we used can cause intestinal hypertrophy in rats. We did not observe this. However, our study was of limited duration (five weeks of diet feeding as opposed to 8–10). We found that the increased villus length of iron deficiency was associated with much of the increase in mucosal reductase activity in iron deficient mice. Changes in villus length were not seen to be caused by Hfe KO although iron deficient feeding induced changes to the same extent in Hfe KO mice as in WT mice. This observation suggests that selective effects on gene expression are caused by Hfe gene deletion and these effects differ, at least in part, from the effects of iron deficient diet feeding. Furthermore, the Hfe gene is not required for the adaptive changes in reductase activity and villus length seen when an iron deficient diet is fed.

CONCLUSION
The Hfe gene product and dietary iron downregulate villus reductase activity in mice. Adaptation in mucosal reductase activity in iron deficient diet fed mice involves increased villus length as well as increases in cellular reductase activity and these changes do not require the Hfe gene.

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